Zeiss Confocal LSM510 Microscope Instructions

No Food or Drink Permitted in the Microscope Room
CRITICAL: The microscope can be seriously damaged by improper care of lenses. So review the following very carefully before turning on the microscope.

Lens care: Jarring a lens in any way can permanently destroy the alignment of the elements within it. Handle lenses very gingerly. Before examining a sample, make sure there is no foreign material on the sample that could contaminate the lens surface. After use, clean lenses while in the nosepiece and only with lens paper. Fold the paper several times and rub it back and forth over the lens surface. Use several pieces of lens paper until there is no longer evidence of oil on the paper. Avoid pressing the lens paper with your finger into the transparent part of the lens. If the lens becomes contaminated with any substance, contact the facilities manager for instructions on cleaning. Don't even think of applying any solvents to the lens surface.

What are the advantages of this imaging system?

This imaging system consists of fluorescence Axiovert200M inverted microscope with 100W mercury , confocal laser scanner and three lasers: powerful 40 mW argon (green) laser with excitation laser lines of 458, 488, 514 nm; HeNe (red) with 543nm laser line; and HeNe (far red, Cy5 etc.) with 633 nm laser line. There is no laser for UV-exited dyes (Hoechst staining, etc). However, UV-exited dyes may be imaged on confocal w/o confocal effect, just to show the contours of the nucleus. The system is controlled by Zeiss LSM software. This imaging system was developed for rapid confocal acquisition and analysis of images. It is especially suitable for FRAP, because one may bleach regions of interest of various shapes. Files may be exported as TIFF or Excel files suitable for importing into other programs.

Turning on the system

Turn on the mercury lamp. Find AttoArc2 box on the lower shelf on your left and switch on the power. Then press and hold the "ignite" button until green light next to this button comes on. On the mercury bulb control unit switch to level 1 (50% of the mercury lamp efficiency). You may regulate the brightness of the bulb by changing the percentage with + and - buttons.

Next, switch on the general power control - a small free-standing box to the left of the microscope. The microscope, the scanner and laser general controls, and the computer will be switched on in the correct order. If the computer is not switched on, use the computer switch to turn it on.

Log on into the system.

Start the LSM510 program. Select Scan New Images (default), click on Expert Mode button.

IN the menu bar select Acquire. In the Acquire menu subset click on Laser button.

Switch on the laser you are going to use. For argon laser set the rheostate to 75%.

Shutting down the system

Move your data to the SC-B41-SSA_SSA_41_G1_SERVER/SSA_G1 /LRBGEIMAGE/YourFolder.This server will be automatically mounted on all the computers of the facility. If you will not see this server mapped on your personal computers, map it yourself. Find red N in the lower right corner of the computer screen, right-click on it. Select Novell Map Network Drive and for the "network path to resource" field, use the following syntax \\lrbgeimage.nci.nih.gov\ssa_41_g1\lrbgeimage.

Wipe the objectives from oil and lower them.

Go to Acquire/Laser control and switch off the lasers. The cooling unit will be running for extra 5-10 min. ***You should not switch off the general control switch until the cooling system stops running.***

Exit the program. If the laser cooling system is still running, you will get a message: "Do not shut down the system, the laser is still running". You can still exit the program, but wait till the cooler stops before proceeding to next step.

Go to Start and select Shut Down. Eventually you will see the message - Now it is safe to turn down the computer. Switch off the system with a general switch. Switch off the mercury lamp.

Turning on the microscope for standard fluorescence

Click on Acquire/ Vis button. This is a setting that allows focusing on the object through the eyepieces.

Click on Acquire/Microscope Control button.

You can switch transmitted light on and off, and control its intensity with a slider.

Select objective in the "Objective" section.

Select the appropriate filter cube in the "Reflector turret" section.

Open and close the fluorescence shutter by checking and unchecking the On box in "Reflected light"section of the window.

Setting up for confocal imaging

Click on Acquire/ LSM button. This is a setting that enables confocal scanning.

Go to Acquire/Config and and select the appropriate scanning procedure. Select Single track. Hit Save/Apply button, select routine from the list. Or create your own and save it as something else.

Go to Acquire/Scan control. Adjust Mode and Channels. Save this routine by hitting Store/Apply button in Config window and storing the settings under the same name. Each time you will change the settings in Acquire/Scan, save the changes, so that new settings could be used in Macros.

Use buttons Find to find the exposure, Fast scan to quickly adjust the brightness. This command ignores Mode settings. Single - single image collected with all the settings set in Mode and Channels. Continuous - continuous imaging with all the settings set in Mode including averaging.

Change the zoom either within Acquire/Scan window, or go to the image window and select. The frame will appear in the Image Window. One can resize this frame, rotate it. The smaller is the scanned region in y, the faster will be the scan.

Doing FRAP
Protocol 1 - for slow exchange rate.

Bring the laser power for argon laser to maximum (100%) in Laser window. You may have to modify it in future, if your specimen will be excessively bleached during imaging.

1. Go to configuration Control and select the appropriate scanning procedure. Select Single track. Hit Save/Apply button, select routine from the list. If you do not have bleach routine saved, open FITC (Cy2) and resave it as * agfpbleach. It will be saved first on the list.

2. Go to Acquire/Scan control. Select Mode. Set Scan Speed to MAX. Select 12 bit acquisition and unidirectional scan.

Go to Channels and set the pinhole to 1. Some people set the pinhole to the max. You will not have confocal effect at this setting but if your cells are moving large pinhole will compensate for shifts of the focus.

Collect the Single image, and adjust zoom appropriately (usually we use Zoom 3 or 4).

Adjust the settings so that you will have no saturated pixels. Bring the laser intensity to 0.2, so that bleaching of your image during regular scanning will be minimal.

3. Go to Bleach control and create bleach settings if you have not done it before, and Save it. Select 30 scans before the bleach. For GFP bleach use 1 iteration, all the argon laser lines (458, 488, 514) to 100%. Define the Bleach region from within the same Bleach control window - press the appropriate

button and the dialog window for ROI will open. Define Region. Select the appropriate tool, then select the region of interest. Add it to the list of ROI. It is advisable to use the standard size of the ROI through all your experiments.

4. Go to Time Lapse. Input the number of scans, which consists of the prebleach scans (30) plus postbleach scans. We use 230 scans, the longer the better. Also define the time interval between scans. If the exchange rate is slow, the time interval can be 1 sec. Alternatively, you may define faster scan in the first few seconds and slower scan later. To set your scan in this way, go to Macro - see below. Click on B button.

5. Go to Macro menu and click Multi-Time acquisition. In the macro window select appropriate routine if it had been saved. Alternatively, define a new routine. The first block should define prebleach scan. The second block should define bleach and post-bleach scan. You can also specify a third block if you want an additional time lapse with different time interval. Each time you change anything in the Scan or Bleach windows, save the changes and then hit the button Re-initialize in MultiTime window. Check carefully whether you have the appropriate ROI selected.

Protocol 2 for rapid exchange rate.

Bring the laser power for argon laser to maximum (100%) in Laser window. You may have to modify it in future, if your specimen will be excessively bleached during imaging.

1. Go to configuration Control and select the appropriate scanning procedure. Select Single track. Hit Save/Apply button, select routine from the list. If you do not have bleach routine saved, open FITC (Cy2) and resave it as * agfpbleach. It will be saved first on the list.

2. Go to Acquire/Scan control. Select Mode. Select 12 bit acquisition and unidirectional scan.

Go to Channels and set the pinhole to 1. Some people set the pinhole to the max. You will not have confocal effect at this setting but if your cells are moving large pinhole will compensate for shifts of the focus.

Collect the Single image, and adjust zoom appropriately (usually we use Zoom 3 or 4).

Select ROI for rapid scanning. We suggest the height of 48 pixels. The smaller the height, the faster will be the scan. The width does not affect the scan speed. Use the ROI of the same size, or at least height for all of your experiments. This will ensure that images will be collected with the same speed.

Set the scan speed to the MAX.

Adjust the settings so that you will have no saturated pixels. Bring the laser intensity to 0.2, so that bleaching of your image during regular scanning will be minimal.

3. Go to Bleach control and create bleach settings if you have not done it before, and Save it. Select 30 scans before the bleach. For GFP bleach use 1 iteration, all the argon laser lines (458, 488, 514) to 100%. Define the Bleach region from within the same Bleach control window - press the appropriate button and the dialog window for ROI will open. Define Region. Select the appropriate tool, then select the region of interest. Add it to the list of ROI. It is advisable to use the standard size of the ROI through all your experiments. We use circle of 25 pix in diameter.

4. Go to Time Lapse. Input the number of scans, which consists of the prebleach scans (30) plus postbleach scans. We use 230 scans, the longer the better. The time interval between scans should be 0. Alternatively, you may define faster scan in the first few seconds and slower scan later. To set your scan in this way, go to Macro - see below. Click on B button.

5. Go to Macro menu and click Multi-Time acquisition. In the macro window select appropriate routine if it had been saved. Alternatively, define a new routine. The first block should define prebleach scan. The second block should define bleach and post-bleach scan. You can also specify a third block if you want an additional time lapse with different time interval. Each time you change anything in the Scan or Bleach windows, save the changes and then hit the button Re-initialize in MultiTime window. Check carefully whether you have the appropriate ROI selected.

Analysing your FRAP data

To get the values click on Mean ROI in Image window. If you used protocol 1, you will be able then select the ROI you used for bleaching and to get the values for exactly the same region. If you used protocol 2, you will have to guess where your bleach ROI was and draw the similar circle in this region. Also you will need to draw the circles of the same size in the region that was not bleached, and in the region with no cells (background). After that Click on Show Table. Then Save Table (it will be exported as text file that can be opened with Excel). If you will click on Save button within the image window, the image will be saved with ROIs that you have drawn. In this way you will always know which regions you analysed.

The pixel values of the table should be normalized as follows:

(bleached spot - background)/ (nonbleached spot - background) x N

N is the average of the values for the last 10 prebleach timepoints.

Collect the FRAP data for 10-20 cells. Plot the normalized values on the same graph. Also plot the average values with standard errors.

Saving your files

First of all, save all your files as LSM files. This format contains all the info about the file and can be opened by LSM510 program. However, to view your files in Photoshop or other program, go to Export and save your files as TIFF or Photoshop files.

Quick way to save overlay and individual channels in any chosen color. Suppose, you have cyan, yellow, garnet and overlay image. Open the file as split xy and click on FILE/EXPORT. Select "Contents of image window single" and appropriate format. The window will be saved as it is - cyan, yellow, garnet and overlay channels on the same image. In Photoshop you may cut out those images, enhance brightness etc. You will not lose any resolution if you are saving the images in this way. The individual image is 512 x 512 pixels, and the Split XY image is 1025 x 1025 pixels.

Saving monochromatic images. After you have opened the file, go to File/Export and check "Monochrome". You will have a choice of the channels, you will be exporting each channel separately.

Exporting images to Metamorph. If you have collected images in a single channel (z-scans, time lapse, FRAP), you are lucky. You may export raw data as tif (8 bit), or as 16 bit tiff. The latter will be opened by Metamorph as 16 bit monochrome.

If you have multichannel image you may save colored overlay image as raw data in tif format (8 bit per channel). After that you may separate colors in Metamorph. Colored overlay images saved as raw 16-bit and 12-bit will not be opened by Metamorph or opened erroneously. You may save individual channels but not overlay as monochrome images at 16 bit. They will be opened by Metamorph.

Batch Export macro is available. The staff may install it for you. Run this macro. Open the database. Select the files you want to export. Select the type of data - raw single image, raw series of images. Select the format - tif, 16 bit tif. Start batch export. Deselect "overwrite files" if the you have several lsm files with the same name, otherwise only one of those lsm files will be exported. Batch export does not allow export of individual channels, only overlays. You have no choice of photoshop format. You cannot export unusual colors, they will be converted to RGB. Also, you will not be able to export 16 and 12 bit overlays, however, if the image is in single channel, you may export it as 12 or 16 bit tif.

Saving only overlay. Open the file as single image. If you have unusual colors, you need to create a special palette for the image and select it before you start exporting. Go to File/Export, select option Full resolution image window single. You may save this image in tif format or Photoshop, or LSM4 RGB.